Retinoic acid receptor β2 and neurite outgrowth in the adult mouse spinal cord in vitro

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Summary

Retinoic acid, acting through the nuclear retinoic acid receptor β2 (RARβ2), stimulates neurite outgrowth from peripheral nervous system tissue that has the capacity to regenerate neurites, namely, embryonic and adult dorsal root ganglia. Similarly, in central nervous system tissue that can regenerate, namely, embryonic mouse spinal cord, retinoic acid also stimulates neurite outgrowth and *RARβ2* is upregulated. By contrast, in the adult mouse spinal cord, which cannot regenerate, no such upregulation of *RARβ2* by retinoic acid is observed and no neurites are extended in vitro. To test our hypothesis that the upregulation of RARβ2 is crucial to neurite regeneration, we have transduced adult mouse or rat spinal cord in vitro with a minimal equine infectious anaemia virus vector expressing *RARβ2*. After transduction, prolific neurite outgrowth occurs. Outgrowth does not occur when the cord is transduced with a different isoform of *RARβ* nor does it occur following treatment with nerve growth factor. These data demonstrate that *RARβ2* is involved in neurite outgrowth, at least in vitro, and that this gene may in the future be of some therapeutic use.

Key words: Retinoic acid receptor β, Neurite outgrowth, Viral vectors, Mouse, Spinal cord

Introduction

The induction of axonal regeneration in the adult central nervous system (CNS) is a major goal in neurobiology. The failure of CNS axons to regenerate under normal circumstances has been attributed to several causes. These include the presence of growth-inhibiting molecules either in the local environment or in the glial scar, a low abundance of neurotrophic factors or the absence of growth-promoting molecules. Attempts to restimulate axon growth in the CNS have generally focused on the first two possible causes. For example, olfactory ensheathing cells and peripheral nerve grafts have been used to provide a permissive environment. In adult rats, olfactory ensheathing cells have induced the restoration of locomotor function in lesioned corticospinal tract axons (Li et al., 1997), and in the presence of peripheral nerve grafts, spinal cord and medulla neurons have extended axons up to 30 mm (David and Agayo, 1981). Following the combination of nerve grafts with the application of fibroblast growth factor, partial restoration of hind limb function has been observed (Cheng et al., 1996).

Neurite growth inhibitors such as Nogo (GrandPre et al., 2000; Chen et al., 2000; Prinjha et al., 2000) are present in myelin and when, in young rats, these inhibitors were neutralized with antibodies, longer axonal extension was observed compared with control animals (Schwab, 1991). A similar treatment has led to the recovery of specific reflex and locomotor functions after spinal cord injury (Bregman et al., 1995). A combination of neurotrophin-3 and these neutralizing antibodies was successful in inducing long distance regeneration of corticospinal tract axons (Schnell et al., 1994), and neurotrophins alone have induced the regeneration of dorsal root sensory axons back into the spinal cord (Ramer et al., 2000). The glial scar is also a component of the inhibitory environment and is composed of extracellular matrix molecules, including chondroitin sulphate proteoglycans. Such molecules are inhibitory to axon growth in vitro (Niederost et al., 1999), and when chondroitinase ABC was administered to the lesioned dorsal columns of adult rats regeneration of both the ascending sensory and descending corticospinal tract axons was promoted (Bradbury et al., 2002).

It is possible, therefore, that neurotrophins may act simply to keep axotomized neurons alive (Kobayashi et al., 1997) and that CNS axons are capable of regenerating, but are normally prevented from doing so by an adverse environment in vivo. Consequently, the environment surrounding the neurons has been a focus of attention in these regenerative studies. However, it is also possible that a combination of approaches, including additional activation of transcription within the nucleus of a damaged neuron, may be required and may ultimately prove successful.

Retinoic acid (RA) can induce nuclear transcription. It is the biologically active metabolite of vitamin A and is present in various tissues of the developing embryo and adult animal, especially the nervous system (Wagner et al., 1992; Horton and Maden, 1995; McCaffery and Drager, 1994; McCaffery and Drager, 1995; Yamamoto et al., 1996; Maden et al., 1998a). In the absence of RA, developing neurons of the CNS do not extend neurites into the periphery (Maden et al., 1996; Maden

et al., 1998b). Conversely, many experiments have shown that when applied to cultured neurons, RA induces both a greater number of neurites as well as increased neurite length (for a review, see Maden, 2001) as well as being capable of dictating their direction of growth (Maden et al., 1998c). RA acts at the level of gene transcription because it is a ligand for two classes of nuclear transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (for reviews, see Kastner et al., 1994; Kliewer et al., 1994). There are three members of each class of retinoid receptor α, β and γ as well as several isoforms of each member, and this diversity may be responsible for the pleitropic effects of RA on cells.

In our previous studies to determine the molecular mechanism of action of RA in neurons, we showed that one of these retinoic acid receptors, namely RARβ2, is the crucial transducer of the RA signal. This conclusion is based on the observations that *RARβ2* is upregulated after RA treatment of embryonic mouse dorsal root ganglia (DRG) neurons and adult mouse DRG neurons and in both of these situations neurite outgrowth is stimulated (Corcoran and Maden, 1999; Corcoran et al., 2000). In addition, a RARβ agonist rather than a RARα or a RARγ agonist specifically induces neurite outgrowth from embryonic DRG neurons (Corcoran et al., 2000). As a result we hypothesized that the absence or below-threshold level of this nuclear receptor in the adult spinal cord may contribute to the failure of this tissue to regenerate axonal projections.

The experiments described here test this hypothesis and demonstrate that by manipulating the genome of the neurons themselves, stimulation of neurite outgrowth can be obtained from non-regenerative adult mouse and rat spinal cord in vitro. Adult spinal cord was transduced with a viral vector expressing the nuclear transcription factor *RARβ2*, and as a result, the normally inert spinal cord was transformed into one that can extend neurites. No neurites are extended in spinal cords transfected with the empty vector, with a mutated vector, with a different RAR isoform or after treatment with nerve growth factor, thereby confirming the specificity of the *RARβ2* effect.

Materials and Methods

Cultures

The spinal cord was dissected from embryonic day 13.5, 10-month-old mice or 3-month-old rats and cut into transverse pieces of about 2 mm. These were cultured in cellagen matrix (ICN Flow), prepared by mixing 1 volume of 7.5% sodium bicarbonate, 1 volume of 10× minimum essential medium (DMEM, Gibco) and eight parts cellagen. The pH was adjusted to 7.5 by dropwise addition of NaOH. Explants were fed every two days. The media consisted of DMEM-F12 with glutamine (Gibco), 6% glucose, GMS-A (a trace element mixture of insulin, transferrin and selenium, Gibco), 10% delipidated serum and all-trans-RA (stock solution, 1×10^{-5} M, Sigma). Transductions were carried out on the day of explant isolation. Equine infectious anaemia virus (EIAV) vectors (1 μl), in the presence of polybrene (4 μg/ml), were injected into the explant during plating. Herpes simplex virus (HSV) vectors were added to the tissue culture media. After increasing times in culture (5, 8 or 10 days), explants were fixed as described below. These increasing times were used with the intention of allowing neurites, if induced, to extend into the cellagen matrix so that at later times longer neurites might be detected.

RT-PCR analysis

RNA was extracted (Trizol, Gibco, RNeasy Mini Kit, Qiagen) and cDNA prepared by the use of a Pharmacia kit as described in the manufacturer's instructions. The primers used were specific for *GAPDH*, *RARβ2* and *RARβ4* (for details, see Corcoran et al., 2000). Polymerase chain reaction (PCR) was carried out for 30 cycles for embryonic spinal cord and 40 cycles for adult spinal cord. Amplification was carried out as follows: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C and extension for 1 minute at 72°C. One fifth of the resultant product was then run on an agarose gel.

Real-time PCR was carried out using the Lightcycler SYBR Green Dye kit and Lightcycler machine (Roche), using *GADPH* and *RARβ2* primers as described above. PCR was carried out for 40 cycles for both *RARβ2*- and *LacZ*-transduced adult spinal cord explants. Amplification was carried out as follows: denaturation for 0 seconds at 95°C, annealing for 5 seconds at 55°C and extension for 10 seconds at 72°C. Levels of *RARβ2* transcription were calculated as a ratio of *RARβ2* expression to *GAPDH* expression.

Viral vector construction and production

Minimal lentiviral vectors have been described recently (Mitrophanous et al., 1999; Mazarakis et al., 2001) and are repeated briefly here. The pONY8.0 series of EIAV vectors were derived from pONY4.0Z by introducing mutations that prevented accessory gene (tat, S2 and rev) expression and prevented expression of the N-terminal portion of gag by insertion of T in the first two ATG codons. pONY8.0G was derived from pONY8.0Z by exchanging the LacZ reporter gene for the enhanced green fluorescent protein (GFP) gene. The central polypurine tract (cPPT), which enhances RNA processing and consequently proviral integration (Charneau et al., 1992; Charneau et al., 1994; Zennou et al., 2000), was inserted into the vector 5′ to the genome to make pONY8.0cZ. The *RARβ2* gene was amplified from pBluescriptRARβ2 and the Flag epitope inserted at the N-terminus in a single polymerase chain reaction using the primers 5′-ACTGCCGCGGGCCACCATG**GACTACAAGGACGACGATGAACAAG**TTTGACTGTATGGATGTTCTGTC-3′ and 5′-ACTGGCGGCCGCTCACTGCAGCAGTGGTG-3′. Underlined bases indicate restriction enzyme sites (*Sac*II and *Not*I respectively), and the bold type indicates bases encoding the Flag epitope. The resulting 1399 base pair product was cloned into *Sac*II/*Not*I-digested pONY8.0c to make pONY8.0cRARβ2.

Vector stocks were generated by calcium-phosphate transfection of human embryonic kidney 293T cells plated on 10 cm dishes using a threee plasmid co-transfection with vector (16 μg), gag/pol (pONY3.1, 16 μg) and envelope (VSV-G, pRSV67, 8 μg) plasmids. DNA for use in these transfections was obtained using Qiagen Maxi-Preps (genomes) or from commercial sources (gag/pol and envelope plasmids). After transfection (36-48 hours), supernatants were filtered (0.45 μm), aliquoted and stored at –70°C. Concentrated vector preparations were made by initial low speed centrifugation 6,000 ***g*** for 16 hours at 4°C followed by ultracentrifugation at 50,000 ***g*** for 90 minutes at 4°C. The virus was resuspended in formulation buffer consisting of sodium chloride (37.5 mM), Tris, pH 7.0 (19.75 mM), lactose (40 mg/ml), human serum albumin (1 mg/ml) and protamine sulfate (5 μg/ml), for 3-4 hours, aliquoted and stored at –70°C. Viral titers were at least 3×10^8 t/u/ml.

Herpes viral vector stocks were prepared exactly as described previously (Lim et al., 1997). The titres used for pHSVRARβ2, pHSVRARβ4 and pHSVLacZ were 5×10^4, 4×10^4 and 5×10 t/u/μl, respectively.

Immunohistochemistry

Explants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and permeabilized with 0.1% TritonX-100 (20 minutes). Blocking was carried out in PBS containing 10% v/v normal donkey serum as appropriate for the secondary antibodies.

Primary antibodies were used as follows: anti-neurofilament NF200 (1:5000, Sigma), anti-Flag (1:500 Sigma) and anti-NeuN, (Neuronal nuclei, 1:100, Chemicon). Antibodies were incubated overnight at 4°C in PBS-10% v/v serum. Samples were washed three times with PBS and then incubated with secondary antibodies: Texas-Red-coupled donkey anti-rabbit IgG and FITC-coupled donkey anti-mouse IgG (1:100 and 1:50, respectively, both from Jackson Immunoresearch) were incubated at room temperature for 2-3 hours. After washing, samples were examined under a confocal microscope.

Western blotting

Protein was isolated as previously described (Corcoran and Ferretti, 1999) from embryonic and adult mouse spinal cord. 10 μg of protein was run on a 5% polyacrylamide stacking gel, then a 10% separating gel. The gel was then semi-dry blotted onto nitrocellulose and processed for antibody staining and visualization (Corcoran and Ferrettti, 1999). The antibody used was a rabbit polyclonal RARβ antibody, a gift of P. Chambon.

HPLC

Retinoids were extracted from approximately 300 mg of adult mouse spinal cord tissue according to the method of Thaller and Eichele (Thaller and Eichele, 1987) by homogenizing the tissue in 1 ml of stabilizing solution (5 mg/ml ascorbic acid, Na_3EDTA in PBS, pH 7.3). The homogenate was extracted twice with 2 volumes of 1:8 methyl acetate/ethyl acetate, with butylated hydroxytoluene as an anti-oxidant, and then dried down over nitrogen. The extract was resuspended in 100 μl methanol, centrifuged at high speed to remove any particulate matter and placed into an autosampler vial for analysis.

Reverse phase HPLC was performed using a Beckman System Gold Hardware with a photodiode array detector and a 5 μm C_{18} LiChrocart column (Merck) with an equivalent precolumn. The mobile phases used were those of Achkar et al. (Achkar et al., 1996), which allows a good separation of the retinoic acids and the retinols. The flow rate was 1.5 ml/min using a gradient of acetonitrile/ammonium acetate (15 mM, pH 6.5) from 40% to 67% acetonitrile in 35 minutes followed by 100% acetonitrile for an additional 25 minutes. Individual retinoids could be identified according to their UV absorption spectra.

Results

Effect of RA on embryonic mouse spinal cord in vitro

Initial experiments were designed to confirm that mouse embryonic spinal cord can respond to RA by extending neurites, as has been demonstrated in other areas of the embryonic CNS (for a review, see Maden, 2001), and that this behavior involves an upregulation of *RARβ2*. The spinal cord was dissected from mouse embryos (E13.5), placed in a cellagen matrix and cultured in 10% delipidated serum. All-trans-RA was added to explants of the spinal cord at three different concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M). After 5 days, the explants were stained with an anti-neurofilament (NF200) antibody and were examined for the presence of neurites. In the absence of RA, the embryonic cord extended some neurites (Fig. 1A). This is thought to occur as a result of the high endogenous content of RA and its precursor, retinol, in the embryonic spinal cord (Maden and Horton, 1995; Maden et al., 1998a). These data are supported by evidence showing that when the endogenous synthesis of RA was inhibited with disulphiram, no neurites were extended (Corcoran and Maden, 1999). When spinal cord explants were treated with increasing concentrations of RA, increasing numbers of neurites were

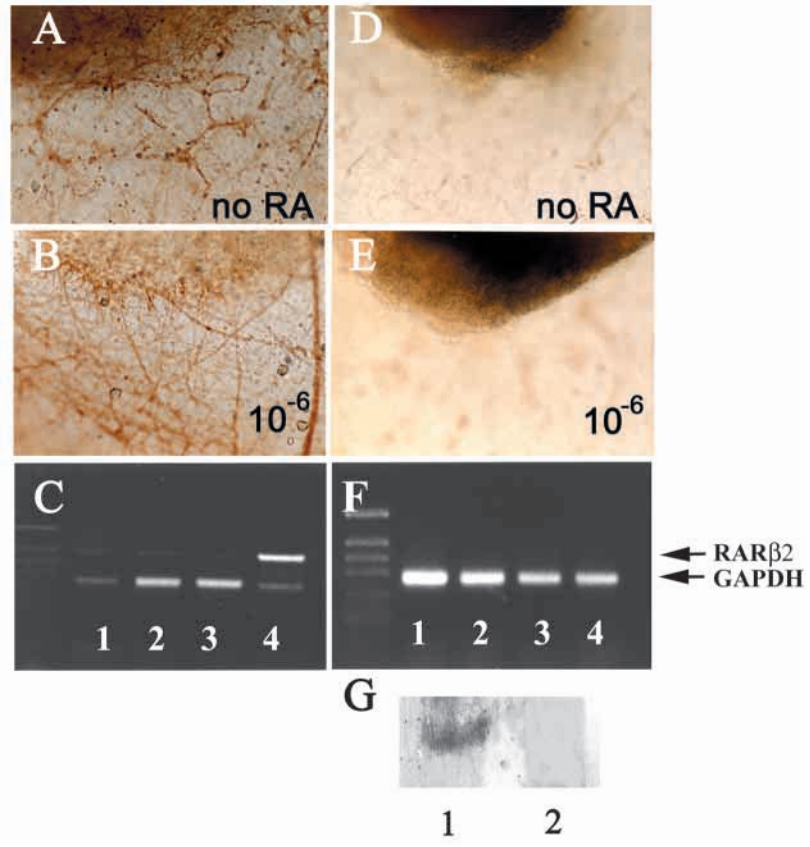

Fig. 1. Induction of neurite outgrowth by retinoic acid. Comparison of the effect of retinoic acid in neurite outgrowth in cultured embryonic day 13.5 mouse spinal cord (A-C) and 10-month-old adult mouse spinal cord (D-F). Pieces of spinal cord were cultured in cellagen in the presence of 10% delipidated serum with or without RA for a period of 5 days. The medium was changed every 2 days. In A and D there is no RA. The embryonic cord extends neurites in the absence of RA, whereas the adult cord does not. In B and E, 1×10^{-6}M RA was added. Many more neurites are extended from the embryonic cord after RA addition, whereas the adult cord is still unresponsive. C and F show RT-PCR analysis of cultured cords to analyze the expression of *RARβ2* after after 5 days. The presence of *GAPDH* was used to indicate equal amounts of cDNA in the samples. (C) Embryonic day 13.5 cord. Lane 1, no RA; lane 2, 1×10^{-8} M RA; lane 3, 1×10^{-7} M RA, lane 4, 1×10^{-6} M RA. RARβ2 is upregulated by RA. (F) Adult cord. Lane 1, no RA; lane 2, 1×10^{-8} M RA; lane 3, 1×10^{-7} M RA, lane 4, 1×10^{-6} M RA. *RARβ2* fails to be upregulated at any concentration of RA. Arrows on the right of F show the position of *GAPDH* and *RARβ2*. (G) A western blot of proteins from embryonic mouse spinal cord (lane 1) and adult mouse spinal cord (lane 2) incubated with a RARβ2 antibody. This part of the blot, at a molecular weight of approximately 45 kDa, confirms the presence of RARβ2 protein in embryonic but not adult cord.

observed with increasing concentrations of RA (data not shown), with the maximal effect at 10^{-6} M (Fig. 1B).

To demonstrate that the induction of neurite outgrowth involved the upregulation of *RARβ2*, reverse transcription followed by PCR (RT-PCR) was performed on cultures after 5 days using the same range of RA treatments as described above (Fig. 1C). This revealed that *RARβ2* is normally expressed in embryonic spinal cord and that it is strongly up regulated after 1×10^{-6} M RA treatment (Fig. 1C, lane 4); the same concentration that gives maximal neurite outgrowth.

Lack of effect of RA on adult mouse spinal cord in vitro

An identical series of experiments was performed using 10-month-old adult mouse spinal cord rather than the embryonic cord. In contrast to the embryonic cord, RA had no effect on neurite outgrowth at any concentration tested, and like the untreated controls (Fig. 1D), these RA-treated adult cords failed to extend any neurites at all (Fig. 1E). Examining the involvement of *RARβ2* by RT-PCR revealed that control adult spinal cord had little or no detectable endogenous levels of this receptor (Fig. 1F, lane 1) and that, unlike the embryonic cord, there was no change in *RARβ2* level in response to RA treatment at any concentration (Fig. 1F, lanes 2-4).

It was hypothesized that the lack of endogenous *RARβ2* expression or the absence of RA-induced *RARβ2* upregulation may be responsible for the inert behaviour of the adult spinal cord. As this observation was central to our hypothesis, we further verified the lack of *RARβ2* expression in the adult cord by the more sensitive light cycler PCR methodology (Fig. 3C) and also by western blotting (Fig. 1G). The former shows the complete absence of any detectable *RARβ2* transcripts in three individual cultured pieces of cord (Fig. 3C, lanes 4-6) and the latter shows that RARβ2 protein is expressed in the embryonic cord (Fig. 1G, lane 1) but not in the adult cord (Fig. 1G, lane 2).

In further support of this hypothesis, we have previously shown that adult DRG that respond to RA by extending neurites also upregulate *RARβ2* (Corcoran and Maden, 1999). To test this RARβ2 hypothesis, we transduced adult mouse and rat spinal cords in vitro with the *RARβ2* gene using both an equine infectious anaemia virus (EIAV) and a herpes viral vector system.

Neuronal transduction and expression of RARβ2 from EIAV vectors

EIAV is a lentivirus, which is a non-human pathogen and has the simplest genome of all lentiviruses, having only three accessory genes. Minimal vectors based on EIAV have been described and transduce both dividing and non-dividing cells (Mitrophanous et al., 1999; Mazarakis et al., 2001). EIAV-based vectors (e.g. pONY8.0Z) cause widespread and efficient transduction in the rat CNS (e.g. striatum, hippocampus, substantia nigra) and in spinal cord in vivo (Mazarakis et al., 2001). To demonstrate that effective transduction can also be obtained in vitro, we used dissociated cultures of rat DRG. Using a pONY8.0GFP virus at a multiplicity of infection (MOI) of 10 our results revealed that approximately 45% of the cells in such a dissociated culture were transduced (Fig. 2A,B). When the mouse *RARβ2* gene, tagged with the Flag epitope, was inserted into the EIAV vector genome (pONY8.0cRARβ2.Flag), expression of the Flag epitope can be detected in both heterologous dividing cell lines (data not shown) and in dissociated primary rat striatal cultures. Colocalization studies with the anti-Flag antibody and an RARβ antibody in striatal neurons indicate that staining is specific for the RARβ2 protein expressed from the pONY8.0cRARβ2.Flag virus (Fig. 2C-E) and that it is expressed specifically in the nuclei of these cells. Like dissociated DRG cells, about 45% of the cells in striatal cultures were transfected.

Induction of neurites in adult spinal cord

The pONY8.0cRARβ2.Flag virus was used next to transduce adult rat and mouse spinal cord explants. Virus (1 μl, minimum titer 5×10^{8} transducing units/ml) was injected directly into the explants, and immunohistochemistry with the anti-Flag antibody demonstrated efficient *RARβ2* gene transfer (up to 60% transduction) of both mouse and rat spinal cords in vitro (Fig. 3A, also Fig. 3F). Individual explants were tested to ensure that they were expressing the *RARβ2* gene using the sensitive light cycler PCR technique. Fig. 3C shows three examples of *RARβ2*-transduced cord explants expressing high levels of *RARβ2* compared with *GAPDH* (lanes 1-3) and three control explants showing no *RARβ2* expression (lanes 4-6).

Following transduction of adult spinal cords with the pONY8.0cRARβ2.Flag, many neurites throughout the cellagen matrix were observed (Fig. 3B). The cells that extended neurites were those that had been transduced, as demonstrated by colocalizing the Flag marker with neurofilament staining (Fig. 3D,E). Fig. 3E shows a high power

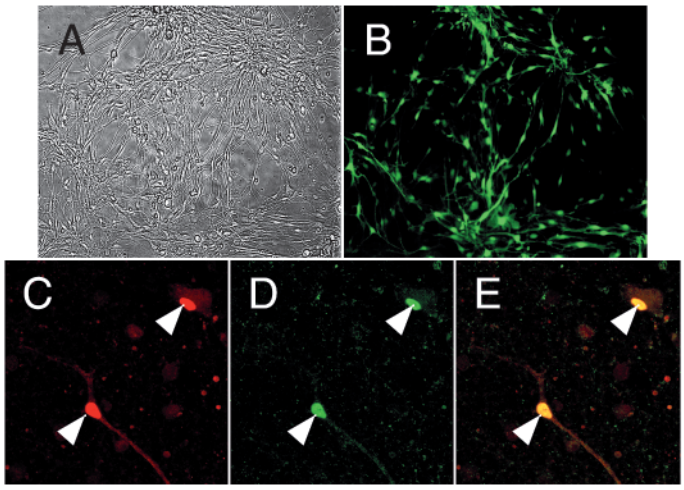

Fig. 2. Neuronal transduction using EIAV. (A,B) Brightfield (A) and fluorescent (B) image of dissociated dorsal root ganglia transduced with pONY8.0G. At a multiplicity of infection (MOI) of 10, 45% of cells in this culture were transduced as shown by GFP fluorescence in B. (C-E) Confocal microscopy images of the same two cells in primary cultures of striatal neurons transduced with the EIAV construct, pONY8RARβ2.Flag. In C a RARβ antibody and in D a Flag antibody were used to visualize transduced cells with Texas-Red- and FITC-coupled secondary antibodies, respectively. In E, the images are merged to show the colocalization (yellow) of the RARβ and Flag antibody staining.

magnification of an explant, with the transduced cells in green, neurofilament staining in red (Fig. 3D) and coexpressing cells in yellow. Although a sizeable region of the explant had been transduced, as shown by the green region in Fig. 3D, neurites only extended from one corner of the explant (red neurites in Fig. 3D). We estimate that about 50% of the transfected cells showed colocalized neurofilament staining (yellow or orange cells in Fig. 3E). Thus all transfected cells do not extend neurites. Perhaps this reflects the variation in levels of RARβ2 expressed in individual cells in the transfected region, which is likely to occur since individual explants varied in RARβ2 levels as a whole (Fig. 3C).

Explants were harvested at 5, 8 and 10 days in vitro. At 5 days, neurites first appeared and at 8 days and 10 days, they extended further into the cellagen matrix and now resembled embryonic cords (cf. Fig. 1D) in both the number and the extent of neurite outgrowth. In Table 1, the data for 5, 8 and 10 days are pooled since we have not quantified the lengths of neurites, and it emphasizes that all explants that were successfully transduced (70% success rate) induced neurite outgrowth from at least part of the explant (Fig. 3D). The typical number of neurites that grew into the cellagen matrix was 30-50 neurites per explant.

The cords that had been transduced with pONY8.0cRARβ2 extended neurites whether or not RA was added to the medium. This could mean that no ligand was required to activate the RARβ2. Assuming a RXR heterodimeric partner is required for RARβ2 activation, then this could potentially occur by the phantom ligand effect (Schulman et al., 1977) in which RAR is activated by a conformational change in its RXR partner.

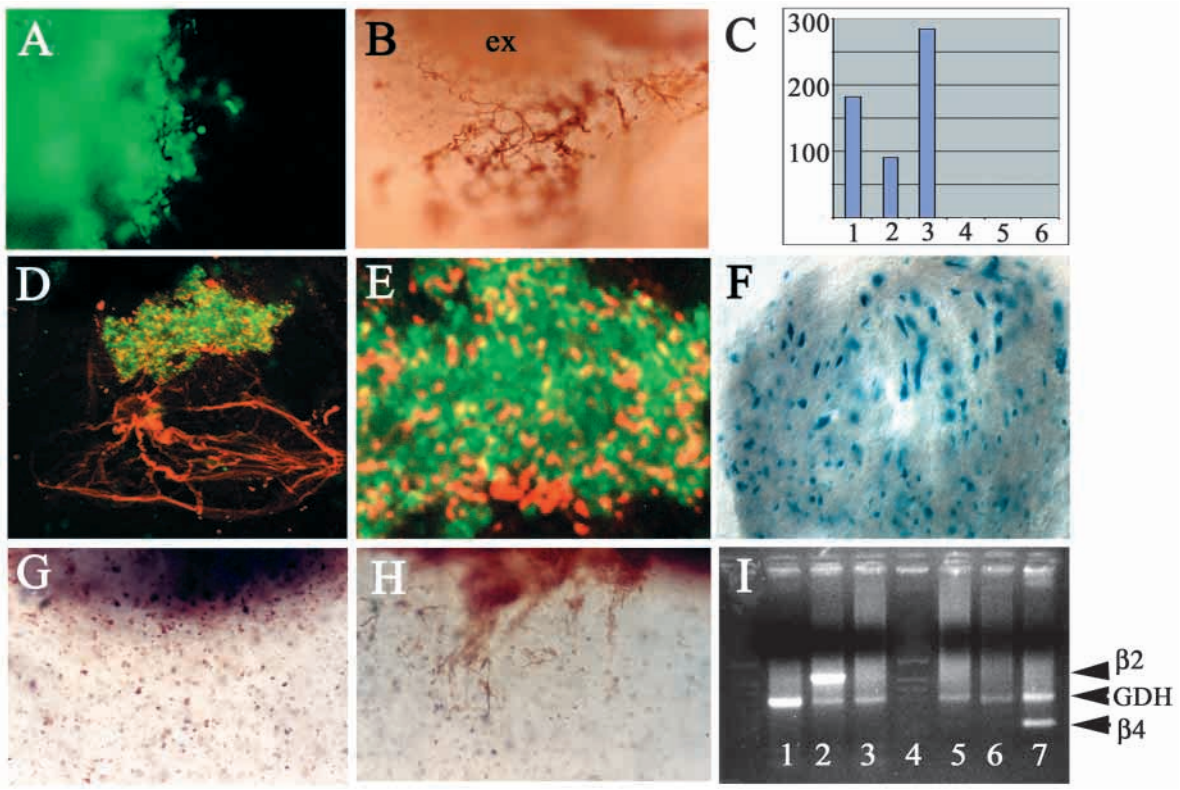

Fig. 3. Viral transduction of spinal cord. (A) Transduction of adult mouse spinal cord with pONY8.0cRARβ2. This culture was analyzed 8 days post-transduction with a FITC-labelled anti-Flag antibody (green). The area of green fluorescence shows a high level of transduction within the cultured cord. (B) The effect of transducing adult mouse spinal cord with pONY8.0cRARβ2. Neurites, stained with an antibody against NF-200 and visualized with diaminobenzidine can be seen growing out from the explant (ex) after 8 days. (C) Light cycler PCR analysis for *RARβ2* expression in three individual *RARβ2* transduced explants (lanes 1-3) and three individual *lacZ* control transduced explants (lanes 4-6) cultured for 8 days. The levels of RARβ2 expression relative to GAPDH are recorded in the blue columns. Explants 1-3 show between a 100-fold and a 300-fold induction of *RARβ2*, whereas explants 4-6 show no expression. **(**D**)** Double-labelled adult mouse cord explant following transduction with pONY8.0cRARβ2. Neurites were stained with NF-200 and visualized with a Texas Red-labelled secondary antibody and can be seen emerging from the lower part of the explant. Transduced cells were visualized with a FITC-labelled anti-Flag antibody (green). Double-labelled cells (yellow) are transduced cells that are also NF-200 positive. (E) Close-up of the explant in D showing green *RARβ2*-transduced cells, red neurofilament-positive cells and yellow double-stained cells. (F) Adult mouse cord transduced with the control virus (pONY8Z) containing the *lacZ* marker gene. After X-gal staining, a good percentage of transduced cells (mostly neurons as judged by their size) is obtained. (G) The effect of transducing adult mouse cord with a herpes virus containing the *RARβ4* gene (pHSVRARβ4). No neurites are induced. (H) The effect of transducing adult cord with a herpes virus containing the *RARβ2* gene (pHSVRARβ2). In contrast to G, neurites are induced in this case, as visualized with NF-200 and diaminobenzidine. (I) RT-PCR analysis of adult mouse cords transduced with either the *RARβ2* gene (pHSVRARβ2) or the *RARβ4* gene (pHSVRARβ4 – lanes 5-7) and analyzed after 4 days. Lanes 1-3, *RARβ2* analysis: lane 1, no virus; lane 2, pHSVRARβ2 transduction showing the presence of *RARβ2*; lane 3, pHSVRARβ4 transduction showing no *RARβ2* expression. Lanes 5-7, *RARβ4* analysis: lane 5, no virus; lane 6, pHSVRARβ2 transduction showing the absence of *RARβ2*; lane 7, pHSVRARβ4 transduction showing the presence of *RARβ4* expression. Arrows to the right mark the position of *RARβ2*, *RARβ4* and *GAPDH*.

Table 1. Neurite outgrowth following viral transduction in the spinal cord

	Numbers of cultures showing neurite outgrowth			
Species	pONY8LacZ	pONY8RARβ2.Flag*Int*-	NGF	pONY8RARβ2
Mouse	0/25	0/10	0/12	19/27
Rat	0/9	–	–	5/7

Summary of the data on adult mouse and adult rat spinal cords transduced with *LacZ* control (column 2), the *Integrase*– controls (column 3), nerve growth factor (column 4) or the *RARβ2* gene (column 5). Cords were cultured for 5, 8 or 10 days in vitro and the results pooled. All the cords that were successfully transduced with *RARβ2* (19 out of 27 for mouse, 5 out of 7 for rat) extended neurites (column 4).

Alternatively it could be that there is enough endogenous RA already in the explant to activate the newly synthesized RARβ2. RA is certainly found throughout the brain and spinal cord of the adult rat and comprises a proportionately higher level of the retinoid pool compared with other tissues (Werner and DeLuca, 2002). In addition, we have detected endogenous retinoids, including RA, in the adult mouse spinal cord by high-pressure liquid chromatography (Fig. 4), although we do not know what happens to these lipophilic compounds during the culturing period. Nevertheless, the potential is there for the RARβ2 to be liganded by RA.

Two control experiments were performed. In one, pONY8.0cZ, a virus encoding the bacterial β-galactosidase gene *LacZ*, was used to transduce parallel cultures. No neurite outgrowth resulted (Table 1), although after exposure to X-gal, clear blue staining was observed (Fig. 3F). Individual explants were taken for light cycler PCR analysis to ensure that no *RARβ2* could be detected in these controls (Fig. 3C, lanes 4-6). In the second control experiment, a virus encoding *RARβ2* with a mutation in the *Integrase* gene, (pONY8.0cRARβ2.Flag.*Int*–), which renders the provirus unable to integrate into transduced cells, was used (Mitrophanous et al., 1999). Again, no expression of the *RARβ2* gene or neurite outgrowth was observed (Table 1).

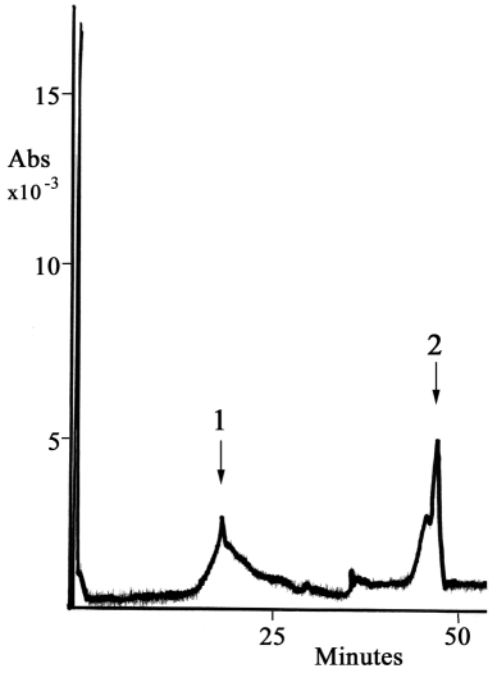

Fig. 4. Presence of retinoids in adult mouse spinal cord. A chromatogram showing two peaks. Peak one has a UV absorbance maximum at 342 nm, and on the basis of its spectrum is identified as all-trans-RA. Peak two has a UV absorbance maximum at 324nm and is identified as all-trans-retinol and/or 13-cis-retinol.

These data have been repeated in another series of experiments in which a defective herpes simplex virus type 1 (HSV-1) vector containing another isoform of the *RARβ* gene, *RARβ4* (pHSVRARβ4), LacZ (pHSVlacZ) or *RARβ2* (pHSVRARβ2) were transduced into adult spinal cord explants. In these experiments, the *RARβ4* isoform provides an additional control as it has been clearly shown not to be involved in neurite outgrowth (Corcoran et al., 2000). Transduction with the pHSVRARβ4 failed to change the behaviour of the cultured adult cord, which remained completely unresponsive in terms of neurite outgrowth (n=12, Fig. 3G). Similarly, the transductions with pHSVlacZ produced no response in the cultured cord, which remained inert (n=12, data not shown). However, neurite outgrowth (eight out of 12 explants) was observed following transduction with the pHSVRARβ2 vector (Fig. 3H). RT-PCR demonstrated that transduction with the *RARβ2* or the *RARβ4* vectors resulted in specific expression of each transcript (*RARβ2*; Fig. 3I, lane 2 and *RARβ4*, lane 7) but not of the other gene (controls in lanes 3 and 6, respectively). In the non-transduced cord neither *RARβ2* nor *RARβ4* were detected (Fig. 3I, lanes 1 and 5).

Finally, since neurotrophins have been used in attempts to induce nerve regeneration in vivo (see Introduction) we wanted to examine whether the same might occur in our explant system. Nerve growth factor (NGF) was chosen because it specifically activates the *RARβ2* gene (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001). However, in the absence of the *RARβ2* gene we expected it to have no effect. Indeed, this was the case, as addition of NGF to our spinal cord cultures did not stimulate neurite outgrowth or extension (Table 1).

Discussion

The results described above provide support for our hypothesis that one of the retinoic acid receptors, RARβ2, plays an important role in the induction of neurite outgrowth in response to RA and further that RARβ2 may be a component of the regenerative pathway that fails to be upregulated in the adult CNS. This hypothesis is based upon several experiments involving either regenerating or non-regenerating neuronal tissues and their response to RA. The regenerating neural tissues we have examined include embryonic mouse spinal cord, embryonic mouse DRG and the adult mouse DRG, all of which respond to RA by upregulating *RARβ2* and extending neurites (Corcoran and Maden, 1999; Corcoran et al., 2000). This neurite outgrowth from embryonic mouse DRG can be stimulated by a RARβ agonist and not by a RARα agonist or a RARγ agonist (Corcoran et al., 2000). By contrast, the non-regenerating adult mouse spinal cord fails to upregulate *RARβ2* in response to RA and fails to extend neurites. Furthermore, NGF-stimulated neurite outgrowth from adult mouse DRG also occurs via upregulation of *RARβ2* and RA synthesis (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001).

Therefore we hypothesized that if the non-regenerating adult

spinal cord cannot upregulate *RARβ2*, and this is the cause of a lack of neurite outgrowth, then introducing the *RARβ2* gene into the adult spinal cord should induce neurite outgrowth. To test this, we transduced adult mouse and rat spinal cords in vitro with the *RARβ2* gene using an EIAV-based vector or a herpes simplex virus vector. With the pONY8.0cRARβ2.Flag virus, a high level of transduction was obtained and a large number of neurites were extended from the explanted adult cords in both mouse and rat. Double immunostaining showed that cells in which the Flag tag (and by implication, RARβ2) was expressed were those that extended neurites. No neurites were observed in several control experiments, which included transfecting with the pONY8.0cZ construct (the virus with no *RARβ2* gene) or the pONY8.0cRARβ2.Flag*Int–* (the virus with the *RARβ2* gene but a defective *Integrase* gene). Experiments with HSV-based vectors gave similar results: neurites were observed from spinal cords transduced with *RARβ2* but not from those transduced with either LacZ or the *RARβ4* isoform. The *RARβ4* provides an additional control for the *RARβ2* transductions, as it is unaffected by RA in embryonic mouse dorsal root ganglia (Corcoran et al., 2000). In further experiments, NGF did not induce neurite outgrowth from adult mouse spinal cords, in contrast to adult DRG (Corcoran and Maden, 1999; Ramer et al., 2000). These results strongly suggest that *RARβ2* is required for neurite outgrowth and that its absence in the adult CNS explains, at least in part, the lack of CNS regenerative capacity. Of course, the experiments were all performed in vitro, and it is not necessarily the case that they are relevant to the in vivo situation. With this major proviso, however, it is conceivable that by introducing the *RARβ2* gene into the adult spinal cord it would be possible to reawaken the regenerative potential of the CNS in vivo.

It will be of great interest to discover the downstream targets of the *RARβ2* gene and therefore the mechanism of genetic induction of neurite outgrowth. It is possible that the targets concerned act purely within the neuron itself to stimulate the formation and outgrowth of a growth cone and ultimately stabilize the new neurite. However, this mechanism being only concerned with events within the neuron itself ignores the data concerning the growth-inhibiting properties of the glial scar and the inhibitory molecules present in the CNS environment surrounding the axon (see Introduction), both of which would serve to inhibit neurite regeneration. So it would be a much more likely possibility that the targets of RARβ2 in the neuron include the induction of genes that signal to the oligodendrocyte to downregulate molecules such as Nogo; the downregulation of genes encoding neuronal cell surface receptors for inhibitory molecules such as the Nogo receptor (Fournier et al., 2001); the induction of genes encoding extracellular enzymes such as chondroitinase (Bradbury et al., 2002) or perhaps matrix metalloproteases, which are known to be induced by RA.

It would also be of interest to examine the relationship between RARβ2-mediated induction of neurite outgrowth and the methods described previously for inducing regeneration in vivo to see whether there are any common mechanisms. For example, is RARβ2 involved in neuronal responses to olfactory ensheathing cells (Li et al., 1997)? With regard to neurotrophin-induced regeneration, it is known that NGF acts via the RA pathway in the adult PNS (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001) as NGF upregulates an enzyme that synthesizes RA, and this RA in turn upregulates *RARβ2*. Although we have shown that NGF does not upreguate *RARβ2* in the adult CNS, it would be interesting to know whether other neurotrophins could act via the RA pathway as a potential general mechanism for neurotrophin action in neurite regeneration. In conclusion, these data support a role for *RARβ2* in the regeneration of neurites in the adult CNS in vitro and indicate that experiments involving in vivo gene therapy with this transcript, perhaps in combination with other treatments, would be worthwhile to test whether any functional recovery of the injured spinal cord could be obtained.

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